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Diagnostic Performance: A Comparative Study of the Leukocyte Differential Count on Four Automated Haematology Analysers

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Summary: The diagnostic performance of the haematology analysers, Technicon H1, Sysmex NE 8000, Coulter STKS and Sequoia Cell Dyn 3000 was determined by comparing their results for automated differential counting with the result obtained for 400 cells by the manual microscopic method. These comparative studies were performed on a group of adults, containing both normal individuals (n = 150) and those affected by various pathologies (n = 113). The number of morphologically false negatives is low for all the systems (from 1.8% for the Cell Dyn 3000 to 6.2% for the NE 8000), while the number of distributionally false negatives was slightly higher (from 4.4% for the Cell Dyn 3000 to 7% for the H1 and NE 8000). The morphological flags, though useful for improving the diagnostic performance of the instruments, show a rather modest sensitivity when taken individually: immature granulocytes/bands from 71% for the STKS to 43% for the NE 8000; blasts from 66.6% for the H1 to 53.3% for the STKS, atypical lymphocytes from 59% for the H1 to 13.6% for the NE 8000; erythroblasts from 42.8% for the STKS to 7% for the NE 8000.

Introduction

In recent years many haematological analysers have been introduced into clinical laboratories, which, in addition to a complete cellular blood count, are capable of providing some new erythrocyte and platelet indices (red cell distribution width, mean platelet volume, platelet distribution width, plateletcrit) and the differential leukocyte count on five or more cell types. Moreover, these instruments generate quantitative or morphological flags to indicate abnormalities. The advantages of the automated differential count over the manual microscopic count using 100 or 200 cells are a greater analytical precision (1–3) (the differential leukocyte count is performed by counting thousands of cells instead of hundreds and is accompanied by excellent accuracy, at least for neutrophils, lymphocytes and eosinophils (4–8)), and a reduction in costs and turnaround time, due to the high throughput. The simultaneous production of "scattergrams" relative to the leukocyte population in association with interpretative flags is also an aid to diagnosis (9–11). The usefulness of the generalized differential count, even when automated, is not universally acknowledged; some authors maintain that when the complete cellular blood count is normal, nor or very few clinical advantages are to be gained from the differential leukocyte count (12). According to other authors, the usefulness of the differential leukocyte count is limited to the follow-up of acute infectious processes, of haematology disorders, of allergic conditions, or to the monitoring of therapies with haematological side-effects, rather than being seen as a screening device (13, 14). We must not forget the initial distrust shown by clinicians towards the introduction of the automated differential count, in favour of the microscopic examination, although the latter's

inaccuracy and imprecision are well-known (15). The purpose of the present study is to evaluate the diagnostic performance (the power to distinguish between pathological and normal conditions) of the differential leukocyte count as performed on four modern haematology analysers (Technicon H1, Sysmex NE 8000, Coulter STKS, Sequoia Cell Dyn 3000), considering the standard NCCLS H20-T (16) as a guideline and analysing simultaneously blood samples taken from the same group of patients. Since these instruments provide flags of morphological suspect: left shift or bands, immature granulocytes, immature granulocytes/bands, blasts, atypical lymph or variant, erythroblasts, it was decided to evaluate the sensitivity, the specificity, the predictive value and the diagnostic efficiency of each flag (17) with reference to a group of patients with a high prevalence of disease.

Materials and Methods

Instruments

The evaluation was performed in the Biochemistry and Haematology Laboratory of Santa Chiara Hospital in Trento (Italy), where 500 complete cellular blood counts and 300 differential leukocyte counts are carried out daily on both in-patients and out-patients. The instruments tested during a two-week period, were Technicon H1 (H1) (Technicon Instruments Corporation, Tarrytown, NY), Sysmex NE 8000 (NE 8000) (Toa Medical Corporation, Kobe, Japan), Coulter STKS (STKS) (Coulter Electronics Hialeah, FL), Cell Dyn 3000 (CD 3000) (Sequoia Turner, Mountain View, CA). All the instruments provide a number of blood count quantities and a five- (NE 8000, STKS, CD 3000) or six- (H1) population leukocyte differential count. The analysers also produce a scattergram for the distribution of the leukocyte populations and a series of morphological and distributional flags. NE 8000, H1 and STKS were equipped with an automatic sampler, whereas the CD 3000 needs manual sampling and a shaking mixer; all the instruments aspirated close-vial blood samples. The analysers were calibrated according to the manufacturers' guidelines and were constantly monitored by an internal quality control system (trilevel commercial blood, inserted three times in the daily routine; moving Bull mean on the analysed samples).

Patients

Blood samples were analysed from 263 subjects of both sexes, aged 18 to 70, 150 of which constituted the control group and were selected according to NCCLS H20-T recommendations. The other 113 patients, who constituted the group with the high prevalence of haematological or inflammatory diseases and whose clinical conditions are specified in table 1, were utilized in the diagnostic performance study. All samples were collected with the aware consent of the donors. Each sample was distributed at the moment of collection (by venipuncture) into five 3 ml K_3EDTA closed tubes (Vacutainer, Becton Dickinson Corporation, Mountain View, CA), which afterwards were randomly assigned to each one of the four instruments and to the reference system. All the specimens were kept at room temperature (18–20 °C) and tested between 30 minutes and four hours after collection. Four blood smears were prepared from each sample within two hours of collection. Two of the four smears were randomly assigned to two operators for the microscopic differential count. The blood smears were fixed and stained using the *Wright* method (Hema-Tek, Ames automatic stain, Miles Ltd, Slough, England).

Tab. 1. Specimen types for the diagnostic performance study.

Clinical condition	Number of samples
Acute inflammation	
– Bacterial infections	17
– Tissue necrosis, surgery	11
– Viral infections	12
Metastatic carcinomas	6
Haematologic disorders	
– Acute leukaemias	9
– Chronic myeloproliferative disorders	7
– Myelodisplastic syndromes	3
– Chronic lymphoproliferative disorders	8
– Aplastic anaemia	4
Healthy subjects	36
Total	113

Drafting of the study

The reference intervals were established on the 150 subjects that made up the control group. For each sample the differential leukocyte count was determined using both the reference method (an average of two 200-cell manual counts on two different smears performed by two morphology experts) and each one of the four evaluated instruments (average of differential counts on two replicates). Frequency distributions were created for each leukocyte population for each used method (reference and tests) and, after removal of outliers (± 3 SD), the central 95 percentile of the distributions was determined. The results obtained from the group of 113 patients were classified as follows: normal, if values of each subpopulation ranked between 2.5 and 97.5 percentile for each system and if no morphological abnormality was present; with distributional abnormality if at least one leukocyte population exceeded the central 95 percentile limits and no morphological abnormality was present. In order to detect the presence of a morphological abnormality in the reference method, the arbitrary limits shown in table 2 were chosen; lower values in percentage of bands, metamyelocytes and myelocytes were added to neutrophils, while atypical lymphocytes (variant) were added to the lymphocytes. A sample was considered morphologically abnormal, according to the results from the tested instruments, if at least one of the following criteria applied:

a) production of one or more morphological flags related to nucleated cells,

b) incomplete differential leukocyte count,

c) flags on the count or count over-range (data exceed the linear or printable range for leukocytes).

Tab. 2. Criteria for the evaluation of the reference differential method. The reference differential was classified as morphologically abnormal if one of the following criteria were met.

Bands	4%
Immature granulocytes	
– metamyelocytes	3%
– myelocytes	2%
– myelo + metamyelocytes	3%
Blasts	0%
Atypical lymphocytes	4%
Erythroblasts	1%

Tab. 3. Comparison of flags from different methods (reference and tests).

Reference	Technicon H1	Sysmex NE 8000	Coulter STKS	Sequoia CD 3000
Bands	left shift	left shift	–	–
Immature granulocytes	IG	imm. grans	imm. grans/bands	IG/bands
Blasts	blasts	blasts	blasts	–
Atypical lymphocytes	atypical	atypical lymphs.	variant lymphs.	–
Erythroblasts*	NRBC	NRBC	NRBC	NRBC
–	LUCs (> 4%)**	–	–	–
–	–	–	–	diff/alert

* NRBC = nucleated red blood cells
** LUCS = large unstained cells

For Technicon H1 the sixth population (large unstained cells) was considered to be an index of morphological abnormality when its value was > 4%; lower values were added to the lymphocytes. Table 3 gives a list of the morphological flags considered for the four instruments. It can be seen that not all the analysers show the same flags. In the Cell Dyn 3000 the generic wording "Diff Alert" is produced by the software version (rev. 1.18) to the signal both distributional and morphological anomalies; in the present work it was always used as a morphological flag. If both distributional and morphological abnormalities were present, the sample was classified as abnormal only for morphology, in agreement with the NCCLS standard H20-T. The results were then classified for each method (reference and tests) as normal, abnormal for distribution, and abnormal for morphology. Then the preliminary analysis was carried out, comparing the average of the two counts from the reference method with the first count of each analyser. In cases of morphological or distributional disagreements, an arbitration by consent was carried out by four morphology experts, using the four smears previously prepared and counting of 800 cells. The subsequent phase of the study consisted of the evaluation of sensitivity, specificity, predictive value and diagnostic efficiency (17) for the single morphological flags relative to the nucleated cells. The analytical sensitivity of the same flags was then evaluated, comparing the percentage of the corresponding cells obtained with the reference method (eye-count differential) in the presence or absence of the instrumental flag.

Results

The reference intervals for the five leukocyte populations in each method (reference and tests) are shown in table 4. The intervals are quite superimposable both among the various instruments and between these and the reference method; the most significant differences are the higher value for the neutrophils related to the 2.5 percentile, and the lower value for the lymphocytes related to the 97.5 percentile on the NE 8000, and the definitely higher value for the basophils related to the 97.5 percentile in the STKS in comparison with the other systems. The definitive results (after the arbitration) for the 113 patients who were classified as normal, abnormal for distribution and abnormal for morphology by each of the evaluated methods, are shown in table 5. Table 6 shows the summarising data on the diagnostic performance. It can be noticed that, on the whole, there were eight distributional false negatives with H1 (7%), eight with NE 8000 (7%), six with STKS (5.3%) and five with CD 3000 (4.4%);

Tab. 4. Determination of reference intervals by reference and test methods.

Quantity	Reference	Technicon H1	Sysmex NE 8000	Coulter STKS	Sequoia CD 3000
Neutrophils (%)	41.2–75.0	41.4–74.0	45.3–74.7	41.3–72.1	40.3–71.6
Lymphocytes (%)	17.7–49.2	16.8–48.0	16.7–44.3	19.8–49.1	18.8–48.4
Monocytes (%)	3.0– 9.0	3.9– 9.4	2.9– 9.0	3.5– 8.3	1.8–10.3
Eosinophils (%)	0.0– 7.8	0.4– 7.9	0.5– 7.3	0.5– 7.9	1.4– 8.6
Basophils (%)	0.0– 1.5	0.3– 1.7	0.3– 1.6	0.2– 2.6	0.4– 1.6

Tab. 5. Comparison of the test methods with the reference method.

Reference	Technicon H1			Sysmex NE 8000			Coulter STKS			Sequoia CD 3000		
	N	D	M	N	D	M	N	D	M	N	D	M
N	32	6	2	32	6	2	34	4	2	33	4	3
D	8	10	5	8	15	0	6	13	4	5	12	6
M	3	6	41	6	17	27	4	9	37	2	9	39

N = normal; D = distributionally abnormal; M = morphologically abnormal

table 7 shows in detail the failed distributional abnormalities in comparison with the reference method for each instrument. There were three morphological false negatives with H1 (2.6%), i.e. those samples that were classified as normal with the test method, even though they had morphological abnormalities when tested with the reference method: two patients with atypical lymphocytes (respectively 6% and 10%) and affected by acute viral infection, and one patient with bands (7%), who had recently undergone an surgical trauma. Then there were six morphological false negatives with NE 8000 (5.3%): three patients with atypical lymphocytes, two of which were affected by acute viral pathology (with 6% and 8% of variant); one patient, who was being treated for acute lymphoblastic leukaemia at present in complete remission, with atypical lymphocytes (5%); one subject with myeloid blasts (< 1%) and affected by refractory anaemia with excess of blasts; one patient with metamyelocytes and bands (6%) and affected by acute bacterial infection; finally one patient from a general surgical ward, with bands (7%). Four morphological false negatives were found with STKS (3.5%): two patients with atypical lymphocytes (8% and 10%) and affected by acute viral infection; one patient with metamyelocytes and bands (6%) and affected by acute bacterial infection, and one patient with bands (7%) from a general surgical ward. Two morphological false

Tab. 6. Final classification of diagnostic performance*.

	Technicon H1		Sysmex NE 8000		Coulter STKS		Sequoia CD 3000	
	Samples	%	Samples	%	Samples	%	Samples	%
Total agreement	83/113	73.4	74/113	65.5	84/113	74.3	84/113	74.3
Partial agreement	11/113	9.7	17/113	15.0	13/113	11.5	15/113	13.2
False normal distribution	8/113	7.0	8/113	7.0	6/113	5.3	5/113	4.4
False normal morphology	3/113	2.6	6/113	5.3	4/113	3.5	2/113	1.8

* The agreement is partial when a different type of abnormality (distributional vs morphological or morphological vs distributional) is found by reference and test methods.

Tab. 7. Analysis of false normal distribution results. Number of samples with abnormalities missed by test methods.

	Technicon H1	Sysmex NE 8000	Coulter STKS	Sequoia CD 3000
Increased neutrophils	2	2	1	—
Decreased neutrophils	1	2	—	—
Increased lymphocytes	2	1	1	1
Decreased lymphocytes	—	1	1	1
Increased monocytes	2	1	2	2
Increased basophils	1	1	1	1
Total	8	8	6	5

Tab. 8. Reliability and frequency of individual flags as detectors of specific abnormalities (samples = 113)*.

Flag	Reference	Technicon H1			Sysmex NE 8000			Coulter STKS			Sequoia CD 3000		
		TP	FP	FN	TP	FP	FN	TP	FP	FN	TP	FP	FN
Bands or left shift	10**	3	17	7	2	4	8	—	—	—	—	—	—
Immature granulocytes and/or bands	28	14	6	14	12	7	16	20	9	8	16	10	12
Immature granulocytes	22**	6	2	16	8	7	14	—	—	—	—	—	—
Blasts	15	10	8	5	9	3	6	8	7	7	—	—	—
Atypical lymphocytes or variant	22	13	6	9	3	5	19	8	8	14	—	—	—
Erythroblasts (NRBC)***	14	4	11	10	1	3	13	6	3	8	2	0	12

TP = true positive; FP = false positive; FN = false negative.
* Each sample can present one or more flags.
** Four samples contemporaneously presents both "bands" and "IG" flag.
*** Nucleated red blood cells

negatives were produced with CD 3000 (1.8%): both patients with atypical lymphocytes (6% and 8%) and affected by acute viral infection. In the 150 subjects who made up the control group, the tested analysers produced morphological flags for nucleated cells, which were not confirmed either by the clinical study carried out on the donors or by the microscopic revision on the four smears. The flags were distributed as follows: two (1.3%) on the H1, both atypical lymphocytes and large unstained cells > 4%; five (3.3%) on the NE 8000, four atypical lymph and one atypical lymphocytes and blasts; two (1.3%) on the STKS, both erythroblasts; twenty-four (16%) on the CD 3000, fifteen diff alert and nine diff alert + immature granulocytes/bands. Table 8 compares the morphological flags identified by the different analysers on the same sample relative to the 61 subjects (all belonging to the patient group) who show morphological abnormalities in at least one of the methods, underlining that in the same sample more than one flag could co-exist. The estimate of sensitivity, specificity, predictive value of the positive and negative tests and of the diagnostic efficiency of the flags was referred to the group of 113 patients; the results are shown in table 9. The analytical sensitivity of the flags was analysed, comparing both the percentage representation and the absolute concentration [concentration = percent × leukocytes (10^9/l)] of the atypical cells detected by the reference method when the instrument produced a relevant flag; the comparative results are shown in figures 1a, b, c, d. For the flags "left shift" and "IG" (immature granulocytes) two instruments produce a single flag (H1 and NE 8000), whereas others produce a mixed flag "IG/bands" (STKS and CD 3000). For the comparability of results, a "mixed" flag was also individualized for H1 and NE 8000, by considering both the "left shift" and "IG" flags to represent "immature granulocytes/bands", irrespective of whether they were produced individually or together.

Tab. 9. Predictive values and efficiency of morphology flagging (samples = 113).

	Technicon H1				Sysmex NE 8000				Coulter STKS				Sequoia CD 3000	
	Immature granulocytes bands	Blasts	Atypical lymphocytes	Erythroblasts	Immature granulocytes bands	Blasts	Atypical lymphocytes	Erythroblasts	Immature granulocytes bands	Blasts	Atypical lymphocytes	Erythroblasts	Immature granulocytes bands	Erythroblasts
Sensitivity %	50.0	66.6	59.0	28.5	43.0	60.0	13.6	7.0	71.0	53.3	36.3	42.8	57.0	14.2
Specificity %	93.0	92.0	93.4	88.8	92.4	96.9	94.5	97.0	89.4	93.3	92.0	97.0	89.0	100.0
Predictive value % of a positive test	70.0	55.0	68.0	27.0	63.2	75.0	37.0	25.0	69.0	53.0	50.0	67.0	61.0	100.0
Predictive value % of a negative test	85.0	94.7	90.4	89.8	84.2	94.0	81.9	88.0	90.5	93.0	87.0	92.0	88.0	89.0
Efficiency %	82.3	88.5	86.7	81.4	80.8	91.1	78.8	85.8	85.0	88.3	81.8	90.5	82.0	89.3

Discussion

In the control group, the reference intervals for the five leukocyte populations are similar to others published for healthy adult populations (18, 19), and are quite superimposable between the reference method and the various tested instruments. Slight decreases were observed for the upper limit of neutrophils on the STKS (−2.9%) and CD 3000 (−3.4%), and of lymphocytes on the NE 8000 (−4.9%), and increases for the lower limit of neutrophils on the NE 8000 (+ 4.1%), relative to the reference method. Another relevant observation is that the upper limit for baso-

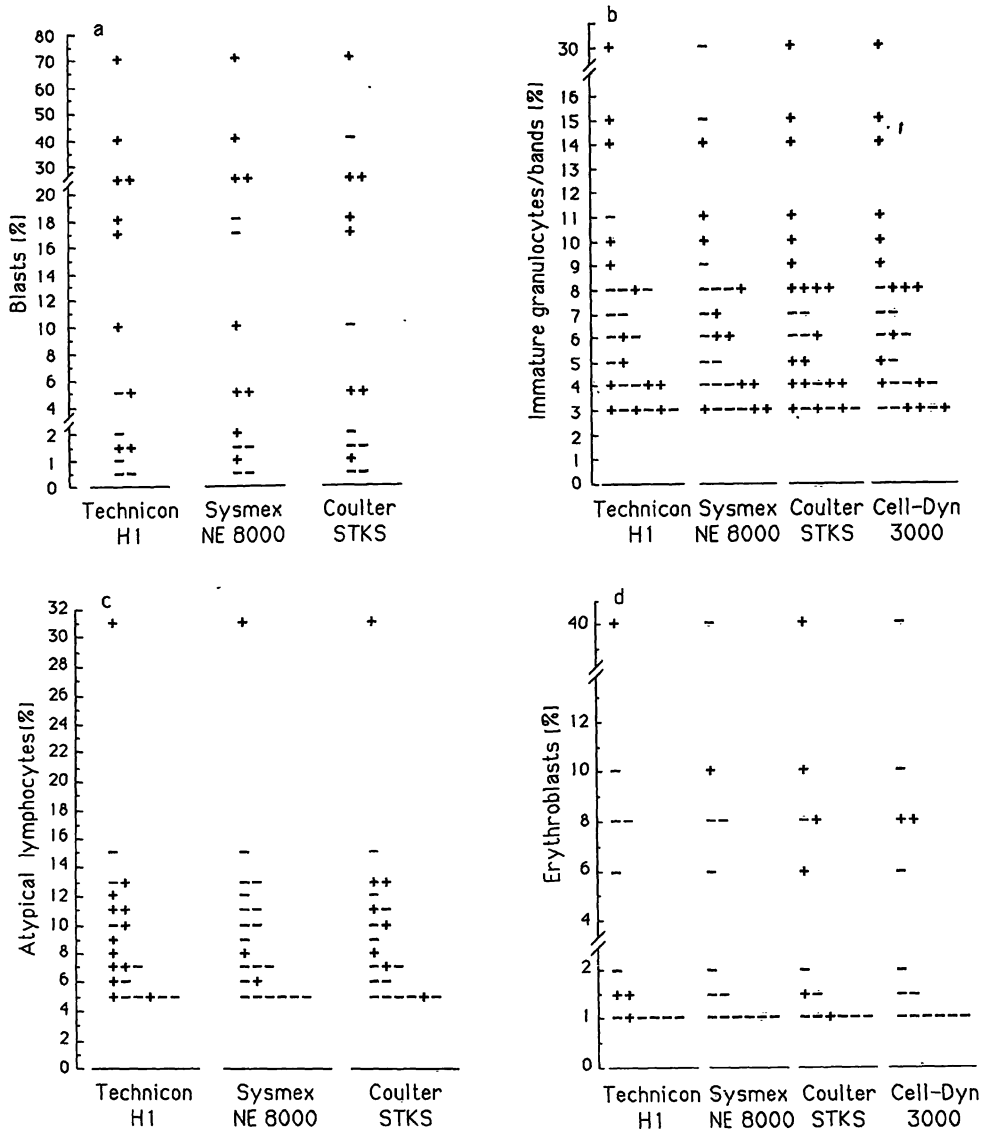

Fig. 1. Comparative results for the analytical sensitivity of individual flags
x-axis: flag present (+) or absent (−), y-axis: percentage of cells found by the manual method;
(a) blasts, (b) immature granulocytes/bands, (c) atypical lymphocytes, (d) erythroblasts.

phils on the STKS is notably higher than for both the reference method (+ 1.1%) and the other analysers (from + 0.9% to + 1%). In this group the instruments produced a low percentage of morphological false positives on the H1 and STKS (1.3%), a slightly higher percentage on the NE 8000 (3.3%), but in a very high one on the CD 3000 (16%). If this last instrument was used routinely to analyse populations with a high prevalence of healthy subjects, it would therefore involve, if compared with the other analysers, a considerable number of microscopic controls, which would not be justified by the clinical conditions of the patient. The study of the diagnostic performance of the differential leukocyte count, using the 113 subjects, shows a good global sensitivity with a very low number of morphological false negatives. Although the distributional false negatives were more numerous, this was of no consequence, because the percentage difference in the various populations was never clinically significant. The agreement between reference method and tests is good: 65.5% (NE 8000), 73.4% (H1), and 74.3% (STKS and CD 3000); the lower agreement observed for the NE 8000 is due to the greater number of samples with morphological abnormalities, not classified as such, but only as a distributional abnormality. The analysis of the flags show that whenever they are used as a generic alarm to direct the operator to perform a microscopic control (as suggested by the manufacturers), they contribute to a remarkable improvement in the diagnostic performance of the instruments. If we consider the flags as detectors of specific abnormalities, however, they show a modest sensitivity:

a) the sensitivity for detecting blasts decreases in the order: H1 > NE 8000 > STKS. This flag, although in all the analysers, tends to become more sensitive as the concentration of blasts increases,

it correlates better with the percentage distribution than with the absolute value (count per litre). H1 shows high analytical sensitivity for the myeloid blasts (no false negatives for values above 1%); whereas the false negatives for lymphoid blasts contained, as determined with the reference method, 5%, 2% and 0.5% blasts. For the other analysers, the variable distribution of false negatives diversifies their response: NE 8000 gave five false negatives for samples containing myeloid blasts, two of these cases containing 17% and 18% blasts; and only one false negative for lymphoid blasts (0.5% with the reference method). STKS reported five cases of false negatives for myeloid blasts (one case with 40% of blasts) and two false negatives for lymphoid blasts;

b) the flags of immature granulocytes or left shift, considered individually (H1 and NE 8000), show a very low sensitivity (values between 30% of H1 and 20% of NE 8000). When they were considered as "IG/bands" flags, the sensitivity decreased in the order: STKS > CD 3000 > H1 > NE 8000. This flag also tends to become more sensitive with increasing concentrations of the young or immature granulocytes, with the exception of NE 8000, which presents two false negatives for reference method values above 15%. As far as the bands are concerned, the reference interval for healthy subjects (obtained through microscope observations) shows a very varied upper limit in the various published studies, ranging from 6% (20) to 6.75% (19) to 8.5% (19) to 10% (21, 22), to 11% (23), up to 12% (24). The cut-off values used in previous researches aimed at evaluating the instrumental flags were also varied, ranging from 5% to 10% (4), to 8% (5), to 11% (23). In the present study, not all the instruments were capable of separating the flag for left shift or bands from that for immature granulocytes. In order to evaluate the analytical sensitivity of the flag (which in this case becomes immature granulocytes/bands), the sum of the percentages of bands and of immature granulocytes detected with the microscope was therefore used as a reference method. In this case the microscope cut-off (bands + metamyelocytes) becomes 7% and agrees with some of the previously mentioned studies. The cut-off of the instrument that shows the greatest analytical sensitivity (STKS) is about 7%, so that only this instrument could have a flag response sufficiently sensitive to replace microscopic analysis. For the other analysers our results agree with those of some authors (4, 25), but are slighly less optimistic than others with reference to H1 (5, 23, 26) or to NE 8000 (5);

c) the flag for atypical lymphocytes shows a sensitivity in the order: H1 > STKS > NE 8000; on the H1 the flag correlates better with the percentage value rather than with the absolute concentration. As regards the other two instruments, the distribution of the results does not show a precise correlation with the percentage or with the concentration of the variants revealed with the reference method;

d) the flag for the presence of erythroblasts is not very reliable in any of the instruments (sensitivity between 42.8% for the STKS and 7% for the NE 8000).

When these analysers are used in hospital laboratories, which give simultaneous access to normal subjects and to those with widely varying pathologies (263 of which were studied by us and gave a significative image), it is important to know the percentage of samples for each instrument that are submitted to microscopic control. If we consider both the distributional and the morphological abnormalities produced by the instruments, this percentage varies from 27.4% (H1 and NE 8000) (a similar percentage has been published for H1 (27)), to 27.0% (STKS) to 36.9% (CD 3000). Whenever the distributional abnormalities are not taken into consideration (and this could be justified by the high level of accuracy and precision in the count of these analysers), this percentage varies from 12.9% (NE 8000) to 17.1% (STKS) to 19% (H1) to 27.4% (CD 3000). However, this last approach must be used with caution, because it risks the exclusion of a reasonable number of samples from the microscopic examination (from 2.2% for the H1, to 3.4% for the STKS and CD 3000, to 6.4% for the NE 8000), in which the distributional abnormality hides a morphological abnormality that is not revealed by the instrument. In conclusion, we can say that the microscopic research, though not totally replaceable by any haematological analyser, is being widely redimensioned, because it is limited to those samples which have count abnormalities or are accompanied by distributional or morphological flags.

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A Multiple Centre Clinical Study of Third-Generation Enzyme Immunoassays for Hepatitis B Surface Antigen and Hepatitis B Core IgM Class Antibody

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Participants

The following institutions were advised to perform tests on clinical samples according to the procedure described in the "Design of the Studies" section:

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Summary: Two new monoclonal antibody-based automated enzyme immunoassays for hepatitis B markers were tested for their clinical performance. The assays specifically detect hepatitis B surface antigen (HBsAg) and hepatitis B core (HBc) IgM antibodies by radial partition fluorescence enzyme immunoassay.

Taken together, the HBsAg and IgM anti-HBc assay show clinical sensitivities of 99.5% and 95.6% and respective clinical specificities of 99.8% and 99.5%, compared with standard ELISAs. The detection limit goes down to 50 PEI units per litre for HBsAg ad subtype and 70 PEI units per litre for ay subtype.

Introduction

Hepatitis B virus (HBV[1]) is an enveloped partially double-stranded DNA virus belonging to the recently designated group of *Hepadnaviridae* (1). More than a quarter of a billion people worldwide carry the virus, which is a leading cause of liver cancer (2). The

[1]) Abbreviations:

HBV	= hepatitis B virus;	FN	= false negative;
HBsAg	= hepatitis surface antigen;	FP	= false positive;
HBcAg	= hepatitis core antigen;	rDNA	= recombinant DNA;
Anti-HBc	= antibody against HBcAg;	TN	= true negative;
HDV	= hepatitis Delta virus;	TP	= true positive;
FDA	= Federal Drug Administration;	PEI	= Paul-Ehrlich Institute.

number of casualties is estimated to 2 millions a year (3). The virion (Dane particle) is 42 nm in diameter and its nucleocapsid 27 nm. The core has an icosahedral form, built up of 180 copies of a 22 000 M_r core protein termed HBcAg. A truncated core protein has different antigenicity and is named HBeAg (4, 5). The lipid bilayer envelope surrounding the core contains the complex antigen determinant called HBsAg (Australia, surface, hepatitis associated antigen), which is composed of carbohydrates, lipids and various proteins. Antigenic subtyping of HBsAg revealed one common determinant to all HBsAg, termed a, and two sets of determinants which mutually exclude each other, namely d or y and w or r. This gives the possible four subtypes: adw, adr, ayw and ayr (6). HBsAg is the first detectable immunoserological marker in the course of a hepatitis B virus infection (7) and a marker of both acute and chronic hepatitis B (8). It usually appears some weeks prior to the onset of hepatitis symptoms, but is cleared quickly in recovering patients. The core antigen (HBcAg) of hepatitis B virus cannot be detected directly since it is masked by the viral envelope, whereas HBeAg can be found in the blood stream. The antibody elicited against HBcAg is another useful marker. IgM anti-HBc has been detected at high concentrations in acute hepatitis B (9, 10) and lower concentrations in chronic hepatitis B (11–15). IgM anti-HBc finds its paramount diagnostic role as a reliable marker for acute hepatitis B infection. Since HBsAg and HBeAg are often cleared quickly (9, 10, 16), IgM anti-HBc covers the diagnostic gap between the disappearance of HBsAg and HBeAg and the appearance of anti-HBsAg and anti-HBeAg antibodies (the so-called core window) (17).

Materials and Methods

The Stratus® IgM anti-HBc and HBsAg assays are rapid qualitative automated procedures intended for use with the Stratus® analysers II and IIntellect (for review of the machines see l. c. (18)). The immunoassay format used is a sandwich method. Both, analyser and kits are manufactured by Baxter Diagnostics Incorporated, Miami, FLA, USA.

For the surface antigen assay, the clinical sample (100 out of 250 µl, either serum or plasma) is pipetted onto the centre portion of a square of glass fibre paper containing pre-immobilized (by complexation with goat anti- IgG Fc mouse antibodies) murine monoclonal antibodies against HBsAg. If HBsAg is present in the sample, binding to this antibody will occur. A different murine monoclonal anti-HBsAg antibody (IgG Fab' fragment) (30 µl) covalently linked to calf intestine alkaline phosphatase is left to react with the bound HBsAg. Application of a substrate/wash solution (1 mmol/l 4-methylumbelliferyl phosphate in 63 g/l diethanolamine buffer pH 9) (20 and 50 µl in two increments) to the centre of the reaction zone results in the migration of unbound conjugate and serum/plasma components away from the reaction centre by radial elution. The fluorogenic reaction is initiated at the same time and measured kinetically via front surface fluorescence. The excitation wavelength is 365 nm and emission is measured at 450 nm. The data analysis is performed by the microprocessor within the analyser (19). The analyser transforms the optical (fluorescence) signal into quantitative mV-readings (mV/min), which are used to report qualitative results, either non-reactive, reactive or highly reactive. Relative reactivity is based on the comparison of sample millivolts to a cut-off value, calculated from the millivolt readings of the 2 positive and 3 negative controls. The negative controls are processed human plasma containing 1 g/l sodium azide. The positive controls are vaccine-grade human plasma-derived hepatitis B surface antigen in a Tris/HCl (pH 7.8) buffer containing: 8.5 g/l sodium chloride, 10 ml/l Brij 35, 0.2 g/l chloramphenicol, 1 ml/l of 4 mg/l clotrimazole in methanol, 7 g/l hydrochloric acid, 80 g/l bovine serum albumin and 1 g/l sodium azide.

The CV tolerance for the positive controls is set at $\leqq 5\%$, and for the negative controls at $\leqq 12\%$. The ratio of the average value of the positive controls to the average value of the negative controls must be > 6.5. If all these criteria are met, the analyser calculates a cut-off (mV/min) using the formula:

$$\text{Cut-off (mV/min)} = \frac{(\bar{x}\ \text{pos. control} - \bar{x}\ \text{neg. control})}{K} + \bar{x}\ \text{neg. control}$$

with K being determined for each kit lot to account for lot-to-lot variations. It is determined by running 1000 negative samples per lot to:

$$K = \frac{\bar{x}\ \text{pos. controls} - \bar{x}\ \text{neg. controls}}{(\bar{x}\ 1000\ \text{neg. controls} + 4\ \text{SD}) - \bar{x}\ \text{neg. controls}}$$

with n ($\bar{x}$ pos. controls) = 90 and n ($\bar{x}$ neg. controls) = 120.

The K-value is determined for each lot by the manufacturer. Samples below the cut-off are classified non-reactive and samples above as reactive. The cut-off is normally situated at about 300 mV/min. Samples yielding values higher then 17 000 mV/min are referred to as highly reactive.

In accordance with an FDA protocol recommendation for HBsAg, specimens non-reactive with Stratus® and the chosen reference are considered negative for HBsAg and need no retesting. Specimens demonstrating reactivity for HBsAg by Stratus® and/or the reference method are re-analysed in duplicate using either of the two methods. If both replicates are non-reactive in the repeat test, the specimen is considered non-reactive by that method. However, if one or both of the replicates are reactive in the repeat test, the specimen is considered reactive for HBsAg by that method. Specimens that are repeatedly reactive (in initial result and repeat result) by one or both methods are confirmed using the confirmatory test for that method (see fig. 1 for a complete outline of the procedure). Thus, the result of the confirmation test determines the final classification. If the confirmatory tests for one sample disagree for Stratus® and the reference method, this sample is referred to as false positive or false negative compared with the result of the reference confirmatory test (see fig. 1, column: final vs. final). The confirmation test for Stratus® HBsAg-assay uses human polyclonal HBsAg antibody to neutralize hepatitis B surface antigen detected in screened reactive and highly reactive samples. Screened positive samples which can be neutralized are confirmed positive, while those which cannot be neutralized are considered false-positive. In the confirmatory assay, a sample aliquot (in the case of highly reactive samples diluted 1/50) is mixed with a negative diluent (equine serum) and with the polyclonal anti-HBsAg antibodies in parallel. If HBsAg is present in the sample, it will be partially removed by the complexation with the polyclonal antibodies. Each aliquot is then added to a tab which contains pre-immobilized monoclonal murine anti-HBsAg as described above. The aliquot which has been premixed with only negative diluent contains uncomplexed an-

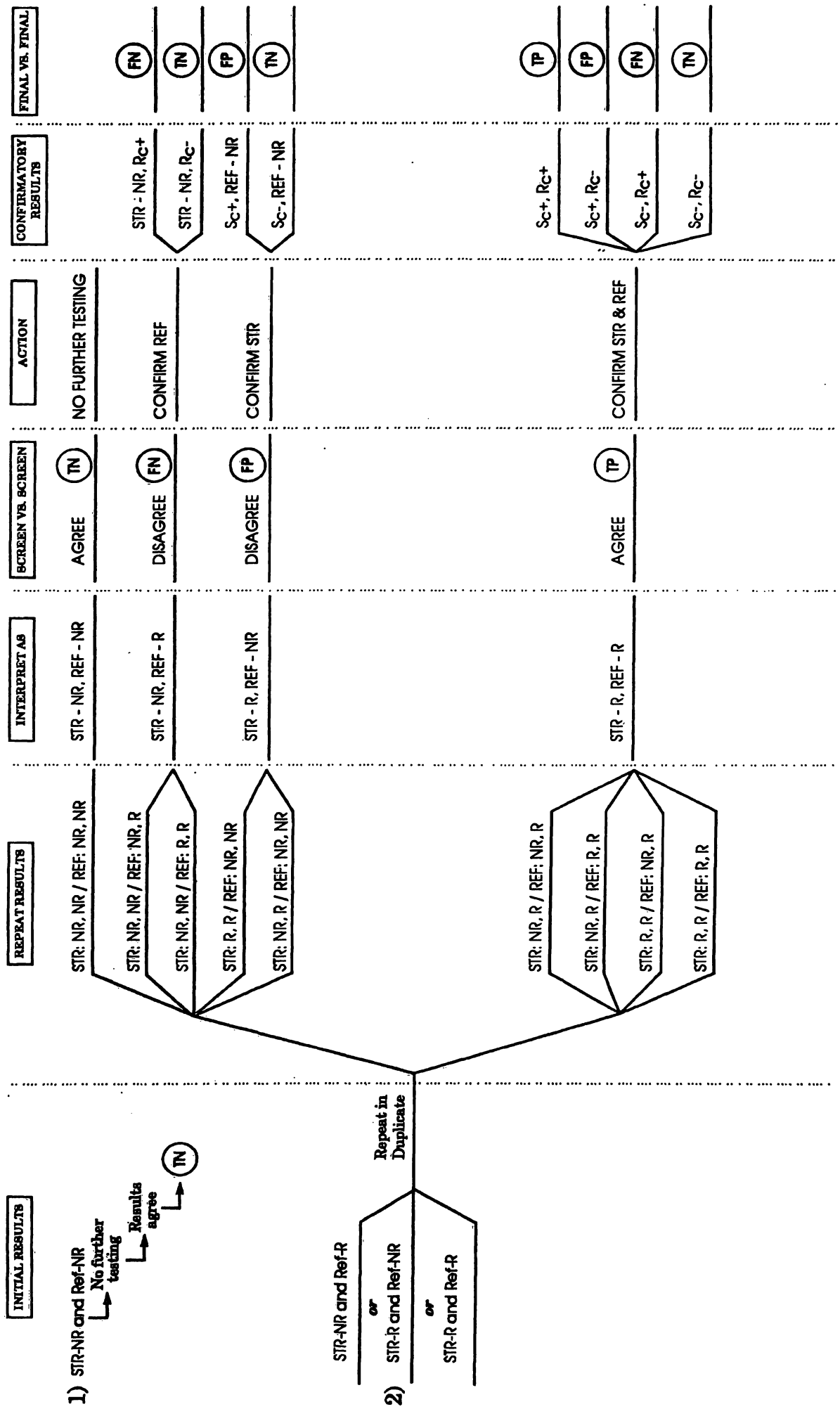

Fig. 1. Testing protocol and interpretation of results of Stratus® HBsAg Monoclonal (STR) and Confirmatory (Sc) assays versus reference screen (REF) and confirmatory (R_c) assays.
NR = – = non-reactive R = + = reactive

tigens which readily bind to available sites on the glass fibre paper tab. This bound antigen is then detected as already described. Samples containing HBsAg and neutralized aliquots show a significantly lower rate of bound conjugate activity than aliquots premixed with negative diluent, whereas HBsAg-negative samples show no difference at all. Reactive samples are considered confirmed if neutralized aliquots show a minimum of 15% reduction in mV/min readings as compared with samples treated with negative diluent. Highly reactive samples have to show at least a 25% reduction.

For the IgM anti-HBc antibody assay, the clinical sample (200 µl) is first automatically diluted 1/625 in sample and positive control diluent (1 g/l gelatine, 20 g/l bovine serum albumin, 150 mmol/l sodium chloride, 1 g/l sodium azide, 1 g/l surfactant in 100 mmol/l Tris/HCl buffer pH 7.6) to exclude possible hook effects (20). IgM present in the sample reacts with a murine monoclonal anti human µ chain antibody immobilized as described above on the tab. Recombinant hepatitis B core antigen (rDNA HBcAg) is applied in solution (40 µl) (composition: 30 g/l bovine serum albumin, 150 mmol/l NaCl, 50 ml/l Tween-20, 1 g/l sodium azide, 100 mmol/l Tris/pH 7.6) forming a sandwich with core-specific IgM antibody which might be present in the sample. In addition, this step removes unbound serum or plasma components from the centre of the tab by radial elution. A murine monoclonal anti-HBc antibody covalently linked to calf intestine alkaline phosphatase in Stratus® conjugate diluent (40 µl) is then added. The formation of the complex is then detected as described above.

As with the surface antigen test, quantitative mV/min data are converted into qualitative results, either as non-reactive, reactive or for re-test. Relative reactivity is based on the comparison of sample millivolts with a cut-off value, calculated from the millivolt readings of the 2 positive and 2 negative controls. The positive controls must lie between 3300 and 6300 mV/min and the negative below 300 mV/min. If these criteria are met, the analyser calculates a cut-off value as follows:

$$\text{Cut-off (mV/min)} = (\bar{x}\ \text{pos. control} - \bar{x}\ \text{neg. control}) \times K + \bar{x}\ \text{neg. control}$$

with K being a kit lot-specific constant with

$$K = \frac{\bar{x}\ \text{cut-off reference prep.} - \bar{x}\ \text{neg. control}}{\bar{x}\ \text{pos. control} - \bar{x}\ \text{neg. control}}$$

The x cut-off value of the reference preparation is the averaged mV/min readings of a preparation of 60 000 PEI units IgM anti-HBc per litre (n ($\bar{x}$ cut-off) = 50, n ($\bar{x}$ neg. controls) = 15, n ($\bar{x}$ pos. control) = 15). Both the $\bar{x}$ cut-off of the reference preparation and the K-value are determined for each lot by the manufacturer.

Patient samples that read > 10% above this cut-off value are reported reactive. Samples ≦ 10% below are considered non-reactive. Patient readings between those limits are reported as re-test. If patient results consistently report as re-test, the status of the patient is to be considered as borderline and should be constantly monitored.

Pipetting and incubation steps with the Stratus® automated analysers are executed in a couple of minutes, so that first results are at hand after 12 minutes for the monoclonal HBsAg and 13 minutes for the monoclonal IgM anti-HBc assay.

Sample storage

Plasma and serum samples obtained in vacutainers were tested immediately or stored at 4 °C for a maximum of one week. Specimens stored for a longer period were kept frozen at −20 °C.

Design of the Studies

All external participants went through a familarization phase with the analyser so as to assure the correct use of the equipment.

Thereafter, 4 kinds of trials were performed:

First, for clinical specificity testing, serum and plasma from apparently healthy donors were analysed for HBsAg according to the FDA protocol outlined above with Auszyme® monoclonal, using its corresponding confirmation test (Abbott Laboratories, North Chicago, IL) as the reference method. In addition, a simple comparison was made between Stratus® and Corzyme®-M (procedure A) (Abbott Laboratories, North Chicago, IL) on healthy blood donors for the determination of IgM anti-HBc.

Second, clinical sensitivity was established by comparing serum or plasma from acute HBV patients in the IgM anti-HBc assay, and from acute HBV patients, chronic HBsAg carriers and patients infected with HDV (implicates co- or superinfection with HBV) in the HBsAg-assay, using the above reference methods.

Third, clinical specificity for various potentially interfering diseases was determined again in comparison with the reference methodology.

Fourth, chronic HBV carriers were tested with the Stratus® IgM anti-HBc assay and with Corzyme-M®, in order to test the claim of both assays for detection of acute hepatitis.

Definitions of concordance and discordance for HBsAg assay (see also fig. 1)

1. Specimens that are initially non-reactive by both methods or are non-reactive by both methods after repeat testing are concordant (true negative = TN).
2. Specimens that are repeatedly reactive by both methods are concordant (true positive = TP).
3. Specimens that are repeatedly reactive by Stratus® and repeatedly non-reactive by the reference method are discordant and defined false positive = FP.
4. Specimens that are repeatedly non-reactive by Stratus® and repeatedly reactive by the reference method are also discordant and defined false negative = FN.

Definitions of concordance and discordance for IgM anti-HBc assay

1. Specimens that are initially non-reactive by both methods are concordant (true negative = TN).
2. Specimens that are reactive by both methods are concordant (true positive = TP).
3. Specimens that are reactive by Stratus® and non-reactive by the reference method are discordant and defined false positive = FP.
4. Specimens that are non-reactive by Stratus® and reactive by the reference method are also discordant and defined false negative = FN.
5. If the Stratus® and/or the reference method result is "re-test", the results are not used for statistical analysis.

Statistical analysis

Clinical sensitivity in %: TP/(TP + FN) × 100
Clinical specificity in %: TN/(TN + FP) × 100

Tab. 1. Detection of purified HBsAg ay and ad subtypes by Stratus® HBsAg assay.

HBsAg subtype ay (10^3 U/l)	mV/min > cut-off	HBsAg subtype ad (10^3 U/l)	mV/min > cut-off
10	10065.9	10	12800.4
5	5732.0	5	6871.4
2.5	3097.5	2.5	3661.7
1	1242.0	1	1575.8
0.5	625.5	0.5	751.4
0.25	251.6	0.25	331.3
0.1	50.8	0.10	92.0
0.07	15.7	0.07	36.0
0.05	−7.3	0.05	9.0
0.03	−44.7	0.03	−22.9

Results are expressed in mV/min above the cut-off value thus specifying the limit of detection as the last concentration with average values above the cut-off (n = 18 for each concentration)

Results

Detectability

In an in-house study, a limit of detection of the HBsAg assay was determined by serial dilution of the Paul-Ehrlich-Institute standards for HBsAg subtypes ad and ay in the following manner. A half-logarithmic dilution of either standard was tested with the Stratus® HBsAg assay. The limit of detection was referred to as the dilution at which the measured mV/min are still above the cut-off value. Each subtype dilution was tested 3 times in duplicate for three different HBsAg assay lots and the average mV/min readings calculated. The detection limit of ay subtype was determined as 70 PEI units per litre and as 50 PEI units per litre for ad subtype (tab. 1).

Clinical specificity

Table 2 shows the results of the specificity studies for the Stratus® HBsAg assay at each of the different trial sites. The resulting overall clinical specificity was 6384/6418 = 99.5%.

Clinical specificity data for the IgM core assay are consolidated in table 3. The resultant overall clinical specificity was 534/536 = 99.5%.

Clinical sensitivity

Table 4 gives the results of the sensitivity studies at each trial site for HBsAg. Overall clinical sensitivity is 681/686 = 99.3%.

Clinical sensitivity data for the IgM core assay are depicted in table 5; the overall clinical sensitivity was 226/237 = 95.6%.

Tab. 2. Clinical specificity data for HBsAg from each trial centre

	Blood Centre Kansas	Baylor College	Conti-nental	North American Biological
TN	1977	104	2305	1998
FP	27	0	5	2
TN/(TN + FP)	98.7%	100%	99.8%	99.9%

Tab. 3. Clinical specificity data for IgM anti-HBc from each trial site

	Blood Centre Kansas	Cliniques St. Luc
TN	499	35
FP	1	1*
TN/(TN + FP)	99.8%	97.2%

* patient with persistent hepatitis B (HBsAg and HBcAg positive)

Tab. 4. Clinical sensitivity data for HBsAg from each trial site

	Blood Centre Kansas	Baylor College	North American Biological
TP	312	271	98
FN	1	3	1
TP/(TP + FN)	99.7%	98.9%	99.0%

Tab. 5. Clinical sensitivity data for IgM anti-HBc for each test centre

	Blood Centre	Miami University	Boston Biomedica
TP	42	109	75
FN	5	3	3
TP/(TP + FN)	89.7%	97.3%	96.2%

Interferences

Samples belonging to various disease states or potential interference risk categories were tested for HBsAg and IgM anti-HBc by both methods (tabs. 6 and 7). HBsAg testing according to the outlined protocol of table 1 identified 15 false positives out of a total of 421 negative reference samples, yielding the clinical interference specificity of 421/436 = 96.6% (tab. 6).

IgM anti-HBc (tab. 7) attained a clinical interference specificity of 425/425 = 100%, since there was only one false negative and no false positive.

Specificity for the discrimination between acute and chronic hepatitis B by IgM anti-HBc assays

Both the Stratus® and the reference method claim to detect acute hepatitis B. When only samples from chronic carriers were examined, the Stratus IgM anti-HBc indicated only 1 patient out of 203 as positive for acute hepatitis B, whereas the Corzyme® mismatched 9 chronic patients into the category of acute hepatitis B (tab. 8).

Discussion

Comparison between the Stratus® HBsAg (screen and confirmatory) assay and the Stratus® IgM anti-HBc assay with a reference ELISA yielded good clinical sensitivity and specificity. If interference specificity (tabs. 6 and 7) is taken into account for calculation of clinical specificity, the following values are obtained:

HBsAg: 6805/6854 = 99.3%,
IgM anti-HBc: 959/961 = 99.8%.

Clinical sensitivity of the HBsAg assay lies at 99.5% with a limit of detection clearly below 100 PEI units per litre. Only the clinical sensitivity of the IgM anti-HBc assay lies below 99% (95.6%), if compared with the reference method. However, IgM anti-HB core is an analyte whose sensitivity might best be calibrated by its correlation to the clinical course of infection and not by searching for a low limit of detection. The core IgM test is used clinically to distinguish acute from chronic or from convalescent infection. In most of the clinical cases it is not necessary to detect extremely low levels of IgM anti-HBc, which may persist

Tab. 6. Clinical specificity data for potentially interfering samples (HBsAg-assay) — compiled data from tests carried out at Blood Centre/Kansas and Baylor College

	Stratus®				Reference method			
	Negative	Positive	Repeatedly positive	Confirmed positive	Negative	Positive	Repeatedly positive	Confirmed positive
Convalescent HBV infection	93	7	2	1	99	1	0	0
Non-A/Non-B hepatitis	11	0	0	0	11	0	0	0
Acute HAV* infection	15	5	1	0	20	0	0	0
Other liver diseases**	13	2	2	1	14	1	1	1
Cirrhosis	12	0	0	0	11	1	0	0
Rheumatoid factor positive	9	1	0	0	10	0	0	0
Systemic lupus erythematosus	8	2	1	0	10	0	0	0
Anti-nuclear antibodies	9	1	0	0	10	0	0	0
Acute *Epstein-Barr* virus infection	7	3	3	3	7	3	3	3
Acute Cytomegalovirus infection	7	3	0	0	10	0	0	0
Renal dialysis	15	5	3	0	20	0	0	0
Multiparous women	19	1	1	0	10	0	0	0
Multiply transfused patients	23	0	0	0	23	0	0	0
Malignancies	24	1	1	0	25	0	0	0
Intravenous drug abusers	23	2	2	0	25	0	0	0
Herpes simplex virus infection	10	0	0	0	10	0	0	0
Male homosexuals	27	3	3	2	27	3	3	1
HIV infection	6	4	2	0	8	2	0	1
Haemophiliacs	29	3	1	0	30	2	0	0
Rubella virus infection	11	0	0	0	11	0	0	0
Syphilis	10	0	0	0	10	0	0	0
Toxoplasmosis	10	0	0	0	10	0	0	0
Total	391	43	22	7	421	13	7	6

* hepatitis A virus
** cholestasis, hepatomegaly, hepatic encephalopathy, hepatoma etc.

Tab. 7. Clinical specificity data for potentially interfering samples (IgM anti-HBc-assay) — compiled data from tests carried out at Blood Centre/Kansas, Miami University and Boston Biomedica

	Stratus®			Reference method		
	Negative	Re-test	Positive	Negative	Re-test	Positive
Convalescent HBV infection	54	0	0	54	0	0
Hepatitis Delta infection	10	1	0	10	1	0
Non-A/Non-B hepatitis	12	0	0	12	0	0
Acute HAV* infection	26	0	0	26	0	0
Other liver diseases**	20	0	0	20	0	0
Cirrhosis	11	0	0	11	0	0
Rheumatoid factor positive	10	0	0	10	0	0
Systemic lupus erythematosus	10	0	0	10	0	0
Anti-nuclear antibodies	20	0	0	20	0	0
Acute *Epstein-Barr* virus infection	26	0	0	26	0	0
Acute Cytomegalovirus infection	20	0	0	20	0	0
Renal dialysis	20	0	0	20	0	0
Multiparous women	20	0	0	20	0	0
Multiply transfused patients	25	0	0	25	0	0
Malignancies	20	0	0	20	0	0
Intravenous drug abusers	25	0	0	25	0	0
Herpes simplex virus infection	10	0	0	10	0	0
Male homosexuals	23	0	2	22	0	3
HIV infection	9	1	0	10	0	0
Haemophiliacs	25	0	0	25	0	0
Rubella virus infection	10	0	0	9	1	0
Syphilis	10	0	0	10	0	0
Toxoplasmosis	10	0	0	10	0	0
Total	426	2	2	425	2	3

* hepatitis A virus
** cholestasis, hepatomegaly, hepatic encephalopathy, hepatoma etc.

Tab. 8. Acute hepatitis B test (IgM anti-HBc); results of chronic hepatitis B carrier samples

	Stratus®			Reference method		
	Negative	Re-test	Positive	Negative	Re-test	Positive
Blood Centre/Kansas	101	0	0	98	0	3
Miami University	23	3	1	20	1	6
Boston Biomedica	75	0	0	74	1	0
Total	199	3	1	192	2	9

long into convalescence or chronic infections. It is more important to see the spike of IgM occurring at the end of the acute phase. Therefore, it is interesting to note that the Stratus® IgM core recognizes only one out of 203 chronic carrier samples as positive, whereas the reference method gives 9 positive results (tab. 8), probably due to detection of trailing IgM anti-HBc.

In conclusion, both new third generation immunoassays (Stratus® HBsAg and IgM anti-HBc) are diagnostic tests for hepatitis B well adapted to the clinical needs. Compared with the traditional ELISA, they offer the advantage of complete automation of the workflow, including print-out of results, built-in control routines, and finally a significantly higher speed.

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